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Development of a TaqMan quantitative reverse transcription PCR assay to detect tilapia lake virus

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ABSTRACT: Tilapia lake virus disease (TiLVD) is an emerging viral disease associated with high morbidity and mortality in cultured tilapia worldwide. In this study, we have developed and validated a TaqMan quantitative reverse transcription PCR (RT-qPCR) assay for TiLV, targeting a conserved region within segment 10 of the genome. The RT-qPCR assay was efficient (mean ± SD: 96.71 ± 3.20%), sensitive with a limit of detection of 10 RNA viral copies per reaction, and detected TiLV strains from different geographic regions including North America, South America, Africa, and Asia. The intra- and inter-assay variability ranged over 0.18–1.41% and 0.21–2.21%, respectively. The TaqMan RT-qPCR assay did not cross-react with other RNA viruses of fish, including an orthomyxovirus, a betanodavirus, a picornavirus, and a rhabdovirus. Analysis of 93 proven-positive and 185 proven-negative samples yielded a diagnostic sensitivity of 96.8% and a diagnostic specificity of 100%. The TaqMan RT-qPCR assay also detected TiLV RNA in infected Nile tilapia liver tissue extracts following an experimental challenge study, and it successfully detected TiLV RNA in SSN-1 (E-11 clone) cell cultures displaying cytopathic effects following their inoculation with TiLV-infected tissue homogenates. Thus, the validated TaqMan RT-qPCR assay should be useful for both research and diagnostic purposes. Additionally, the TiLV qPCR assay returns the clinically relevant viral load of a sample which can assist health professionals in determining the role of TiLV during disease investigations. This RT-qPCR assay could be integrated into surveillance programs aimed at mitigating the effects of TiLVD on global tilapia production.

KEY WORDS: Tilapia · *Oreochromis* spp. · Tilapia lake virus · *Tilapia tilapinevirus* · TaqMan · Quantitative PCR · Diagnostic accuracy

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1. INTRODUCTION

Tilapia lake virus disease (TiLVD), caused by tilapia lake virus (TiLV), is a contagious disease in cultured and wild tilapia (*Oreochromis* spp. and their hybrids). TiLVD often causes mass mortality in naive fish populations (Tattiyapong et al. 2020), with clinical signs such as lethargy, anorexia, and abnormal swimming behavior (Surachetpong et al. 2020). Further, infected fish usually display gross lesions including exophthalmia (protruding eyes), skin darkening, ulcerated or hemorrhaged skin, and ascites (Eyngor et al. 2014, Dong et al. 2017, Surachetpong et al. 2017, 2020). TiLV is a single-stranded, negative-sense RNA virus with 10 genomic segments (Eyngor et al. 2014, Bacharach et al. 2016, Surachetpong et al. 2017) encapsidated within enveloped virus particles ranging from 50 to 100 nM in diameter (Eyngor et al. 2014, Ferguson et al. 2014, del-Pozo et al. 2017, Surachetpong et al. 2017). TiLV is the sole species (*Tilapia tilapinevirus*) in the family *Amnoonviridae* (Adams et al. 2017).

Since the first report of TiLV in Ecuador and Israel (Eyngor et al. 2014, Ferguson et al. 2014), the virus has been detected in tilapia species in 15 other countries: USA (Al-Hussinee et al. 2018), Mexico (WOAH 2022), Colombia (Contreras et al. 2021), Peru (Pulido et al. 2019), Egypt (Nicholson et al. 2017), Israel (Eyngor et al. 2014), Uganda and Tanzania (Mugimba et al. 2018), India (Behera et al. 2018), Bangladesh (Chaput et al. 2020, Debnath et al. 2020), Thailand (Dong et al. 2017, Surachetpong et al. 2017), Chinese Taipei (WOAH 2022), Malaysia (Amal et al. 2018), Philippines (WOAH 2022), Vietnam (Tran et al. 2022), and Indonesia (Koesharyani et al. 2018). Although TiLVD has not been fully investigated, high morbidity and mortality associated with infections have been described in tilapia species, including Nile tilapia *O. niloticus*, red hybrid tilapia (*Oreochromis* spp.), mango tilapia *Sarotherodon galilaeus*, redbelly tilapia *Tilapia zilli*, blue tilapia *O. aureus*, and wild tilapia *Tristamella simonis* (Eyngor et al. 2014, Ferguson et al. 2014, Surachetpong et al. 2017, Tattiyapong et al. 2017, Mugimba et al. 2018). TiLV is also known to cause disease in other species experimentally infected by intraperitoneal injection, including red hybrid tilapia (Tattiyapong et al. 2017), gray tilapia (*O. niloticus* × *O. aureus*) (Mugimba et al. 2019), Mozambique tilapia *O. mossambicus* (Waiyamitra et al. 2021), and zebrafish *Danio rerio*, although zebrafish exposed by immersion did not similarly succumb (Rakus et al. 2020). In other experimental studies, giant gourami *Osphronemus goramy* were prone to TiLV infection, while warmer-water fish species did not develop the disease (Jaemwimol et al. 2018). An additional experimental study, again by injection, also suggested the susceptibility of ornamental African cichlids (*Aulonocara* spp.) to TiLV with high mortality, clinical signs, and histopathological findings similar to the infected tilapia (Yamkasem et al. 2021). To date, the complete genome sequences of TiLV from Thailand, Ecuador, Israel, Peru, USA, and Bangladesh have been deposited in the National Center for Biotechnology Information GenBank database (Bacharach et al. 2016, Surachetpong et al. 2017, Al-Hussinee et al. 2018, Pulido et al. 2019, Subramaniam et al. 2019, Ahasan et al. 2020, Chaput et al. 2020, Debnath et al. 2020). Genetic analysis of TiLV sequences originating from different countries revealed that the Israeli TiLV and TiLV isolated from Asia and South America shared a high sequence identity of 95–99% (Surachetpong et al. 2017, Al-Hussinee et al. 2018). TiLV shares some common characteristics with rapidly evolving negative-sense RNA viruses (e.g. orthomyxoviruses), and thus, there is concern that genetic variation among TiLV strains may affect the sensitivity of current molecular assays. Thus, there is a need to develop diagnostic methods that could be applied to detect various TiLV isolates.

A number of diagnostic methods have been utilized for the detection of TiLV in fish tissues: (1) molecular assays including reverse transcriptase PCR (RT-PCR) (Eyngor et al. 2014, Dong et al. 2017, Kembou Tsofack et al. 2017, Mugimba et al. 2018), real-time RT-quantitative PCR (RT-qPCR) (Kembou Tsofack et al. 2017, Tattiyapong et al. 2018, Waiyamitra et al. 2018), and RT loop-mediated isothermal amplification (RT-LAMP) (Yin et al. 2019, Phusantisampan et al. 2020); (2) virus isolation in susceptible cell lines (Eyngor et al. 2014, Kembou Tsofack et al. 2017, Behera et al. 2018), (3) *in situ* hybridization (Bacharach et al. 2016, Dong et al. 2017, Behera et al. 2018); and (4) immunohistochemistry (Piewbang et al. 2021). Among these techniques, the RT-PCR, semi-nested RT-PCR, nested RT-PCR, and RT-qPCR assays have been commonly used for the detection of TiLV, which all target segment 3 of the virus. However, none of these diagnostic assays have been fully validated for the detection of TiLV from different geographic locations. The objective of the current study was to develop and validate a TaqMan RT-qPCR assay for the detection of TiLV in RNA extracts derived from fish tissues during field outbreaks and laboratory challenge studies, as well as cell cultures displaying cytopathic effects.

2. MATERIALS AND METHODS

2.1. *In silico* TaqMan RT-qPCR primer and probe design

Eight TiLV genome sequences retrieved from GenBank were aligned by segment in MAFFT (Katoh & Toh 2008) using default settings. The alignments for each of the 10 segments were imported into Geneious R10 to generate a consensus sequence with the threshold set to 100%. The consensus sequences for each segment were then individually imported into PrimerExpress v2.0 to design primers and hydrolysis probes using default settings. They were scrutinized to determine the primer and probe combination with the lowest penalty value.

2.2. Generation of TiLV complementary RNA standards

An endpoint RT-PCR reaction using a Qiagen OneStep RT-PCR Kit was carried out in 30 µl volumes containing 0.8 µM of each primer (TiLVstdF and TiLVstdR), 0.4 µM of dNTP mix, 4.8 µl of nucleic acid template, 1.2 µl of enzyme mix, 6 µl of 5× buffer, 6 µl of 5× Q-solution, and 8.4 µl of molecular-grade water. The reaction was carried out using a SimpliAmp thermal cycler (Applied Biosystems) using the following conditions: 50°C for 30 min and 95°C for 15 min; followed by 30 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 30 s; and a final elongation at 72°C for 5 min. The amplified product was subjected to electrophoresis on a 1% agarose gel stained with ethidium bromide. The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen) and cloned using a TOPO® TA Cloning® Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Recombinant plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen) and linearized using the restriction enzyme *Not*I (New England Biolabs). *In vitro* transcription was carried out with 1 µg of linearized plasmid DNA using an Ambion® MAXIscript® T3 *In Vitro* Transcription Kit (Invitrogen) followed by DNase treatment and clean-up using RNeasy columns (Qiagen). The amount of viral complementary RNA (cRNA) transcripts was determined by fluorometry using a Qubit RNA Broad Range (BR) Assay Kit (Invitrogen) and a Qubit 2.0 fluorometer and converted to molecular copies using the formula described by Krieg (1990). The cRNA stock was then serially diluted 10-fold using nuclease-free water and stored at −80°C until use.

2.3. Detection of TiLV RNA by the TaqMan RT-qPCR assay

The RT-qPCR assays were carried out in triplicate, using TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems), in 20 µl volumes containing 0.9 µM of each primer (TiLV-F and TiLV-R), 0.25 µM of probe (TiLV-P), 4 µl of nucleic acid template or RNA standards, 5 µl of 4× universal RT-qPCR mix, and 8 µl of molecular-grade water. The VetMAX™ Xeno™ Internal Positive Control was added into the fourth well of every sample, containing 0.8 µl of 25× VetMAX™ Xeno™ Internal Positive Control - VIC™ Assay (Applied Biosystems), 1 µl of VetMAX™ Xeno™ Internal Positive Control RNA (Applied Biosystems), 4 µl of nucleic acid template, 5 µl of 4× universal RT-qPCR mix, and 9.2 µl of molecular-grade water. In addition, 50 ng of TiLV-negative tilapia RNA was added to the RT-qPCR reactions for RNA standards. The reaction mixtures were loaded in 96-well polypropylene plates (Applied Biosystems) sealed with 50 µm polyolefin film (Applied Biosystems), and at least 3 no-template negative controls (molecular-grade water) were included. Reactions were carried out in a QuantStudio 5 Real-Time PCR System (Applied Biosystems) using the fast protocol thermocycling conditions: 50°C for 5 min and 95°C for 20 s; followed by 40 cycles at 95°C for 3 s and 62°C for 30 s. The result was interpreted as positive if the calculated cycle threshold (Ct) from the 6-carboxy-X-rhodamine (ROX)-normalized 6-carboxyfluorescein (FAM) signal exceeded the threshold assigned by the Applied Biosystems software. As specified by the manufacturer, a Ct value returned by the VetMAX™ Xeno™ Internal Positive Control (IPC) assay of between 29 and 33 indicates that the sample is free of PCR inhibitors.

2.4. Estimation of the TiLV TaqMan RT-qPCR assay parameters

Triplicate 10-fold dilutions of the TiLV cRNA standard (10^7–10^1 copies) were used in each of the 19 experiments (plates) to estimate the correlation coefficient (R^2), *y*-intercept, slope, efficiency, dynamic range, analytical sensitivity, repeatability, and reproducibility of the RT-qPCR assay as previously described (Clark et al. 2018, Stilwell et al. 2018). The RT-qPCR assay was carried out based on the reactions and methods described in Section 2.3. The RT-qPCR assay limit of detection (LOD or analytical sensitivity) was defined as the lowest dilution at which

50% of positive samples (wells) were detected (OIE 2021). The coefficient of variation (CV% = [SD/mean] × 100%) for intra-assay (repeatability) and inter-assay (reproducibility) variability were calculated from the mean and SD of the Ct values within (repeatability) using either the data generated from a single representative RT-qPCR plate (cRNA standards [10^7–10^1 copies] in triplicate) or among (reproducibility) the 19 plates. For the analytical specificity, the RT-qPCR assay was tested against RNA extracts from infected tissues or isolates in cell culture supernatant including an orthomyxovirus (infectious salmon anemia virus), a betanodavirus (red-spotted grouper nervous necrosis virus), a picornavirus (clownfish picornavirus), and a rhabdovirus (infectious hematopoietic necrosis virus).

2.5. TiLV challenge study

A TiLV challenge study was performed for the purpose of generating known positive and negative control tilapia (liver) samples for the development and validation of the TiLV RT-qPCR assay. Sixty juvenile Nile tilapia were obtained from a commercial producer in Florida, USA. They were weighed (mean = 54.5 g, SD = 10.4 g) and acclimated for 30 d in a 567 l tank receiving single-pass dechlorinated municipal water maintained at 25.5 ± 0.5°C. Water flow-rate was set such that complete exchange occurred 4 times per hour and the tank was supplemented with multiple airstones. Water quality parameters (pH, ammonia, nitrite, hardness, dissolved oxygen) were recorded once a week using a Fish Farming Test Kit Model FF-1A (Hach) and a portable dissolved oxygen meter (Hach HQ30D). No ammonia or nitrite was detected, the pH consistently measured 7.0, the average total hardness was 164.4 mg l^{-1}, and the average dissolved oxygen was 6.6 mg l^{-1}. Fish were maintained at a 12:12 h day:night photoperiod and fed 4% of their body weight per day with a commercial tilapia pellet diet.

After the 30 d acclimation period, 50 tilapia were haphazardly assigned to 1 of 6 tanks (84 l capacity). Husbandry continued as described above during the acclimation period. Fish in the treatment groups received either 200 µl (high dose) or 100 µl (low dose) of TiLV supernatant with a viral titer of 3.05 × 10^5 $TCID_{50}$ ml^{-1} (methods described below) by intracoelomic (IC) injection. The experimental infection included duplicate high and low dose treatment tanks (10 tilapia $tank^{-1}$) as well as a single control tank (receiving cell culture supernatant without virus) for both the high and low dose treatments (5 tilapia $tank^{-1}$). Fish were monitored for external lesions and behavioral abnormalities for 22 d post virus exposure. Daily mortalities were weighed and necropsied to obtain liver tissues for virus isolation and RNA extraction for testing against the TiLV TaqMan RT-qPCR assay (methods described below). Surviving tilapia at 22 d post virus exposure and the unexposed fish (negative controls) were euthanized with an overdose of buffered tricaine methanesulfonate (1000 mg l^{-1}) and processed for virus isolation and RNA extraction to be tested using the TiLV TaqMan RT-qPCR assay.

The TiLV isolate (WVL18053-01A) used for injection has been described previously (Al-Hussinee et al. 2018) and was prepared from a frozen stock inoculated into a 175 cm^2 flask containing confluent striped snakehead (SSN-1; E11 clone) cells. The SSN-1 cells were maintained at 25°C and grown in L-15 media (Leibovitz; Gibco) containing 10% fetal bovine serum (FBS; Gibco) with 1× antibiotic/antimycotic (AA; Gibco), resulting in a concentration of 100 IP penicillin ml^{-1}, 100 µg streptomycin ml^{-1}, and 0.25 µg amphotericin B ml^{-1}. After the cytopathic effect (CPE) was complete, the supernatant was clarified by centrifugation at 5000 × *g* (20 min at 10°C). The clarified supernatant was then used for IC injection as well as to determine the TiLV titer by $TCID_{50}$ endpoint analysis using the Reed-Muench method (Reed & Muench 1938). The viral titer was determined by performing 10-fold dilutions of the clarified supernatant onto replicate wells (5 replicates $dilution^{-1}$) of a 96-well plate (200 µl $well^{-1}$) containing confluent SSN-1 cells.

The presence/absence of viable TiLV in the liver tissues of dead fish and fish surviving 22 d post virus exposure (including controls) was evaluated using standard virological methods (Ganzhorn & LaPatra 1994). Tilapia were necropsied to obtain liver tissue samples for virus isolation and RNA extraction for testing against the TiLV TaqMan RT-qPCR assay (see below). For virus isolation, each liver tissue sample was diluted 1:25 in L-15 media containing 2% FBS and then homogenized at high speed with a stomacher (Seward stomacher 80, Biomaster Lab system) for 30 s. The liver tissue homogenates were then clarified by centrifugation at 5000 × *g* (20 min at 10°C) to pellet cellular debris. The clarified supernatant was added to an equal volume of L-15 media containing 2% FBS and 2% AA (Gibco) to make a final dilution of 1:50. The presence/absence of TiLV in the clarified tissue homogenate samples was assessed by inoculating each sample onto replicate wells (5 replicates

sample^{-1}) of a 96-well plate (200 µl well^{-1}) containing confluent SSN-1 cells. The plates were incubated at 25°C and observed daily for CPE for 14 d, at which time blind passages were performed on all samples not showing CPE. After an additional 14 d, all blind-passaged samples were scored. Supernatants from all samples that resulted in CPE and those that did not result in CPE on the initial passage or after the blind passage were tested using the TiLV TaqMan RT-qPCR assay as described below.

Liver tissue and cell culture supernatant samples generated during the challenge study were subjected to RNA extraction using an RNeasy Mini Kit following the manufacturer's instructions (Qiagen). The RNA concentration of each sample was measured using a Qubit 2.0. Samples were diluted to 12.5 µg µl^{-1} for use in the downstream TiLV TaqMan RT-qPCR assay.

2.6. Estimation of TiLV TaqMan RT-qPCR assay diagnostic sensitivity and specificity

The diagnostic sensitivity and specificity of the TiLV TaqMan RT-qPCR assay were determined by evaluating its performance on RNA tissue extracts from reference populations of fish defined by their TiLV exposure status. The proven-positive reference group included fish that had received an IC injection of TiLV (high and low dose treatment groups, N = 38; see Section 2.5) and fish derived from field outbreaks of TiLVD that had previously been confirmed to be positive by another RT-PCR assay (described below). The proven-negative reference group (not exposed to TiLV) included the control fish from the challenge study (N = 10) and fish from a health inspection of apparently healthy Florida-farmed Nile tilapia fingerlings (N = 175) that had previously tested negative by conventional TiLV RT-PCR (Eyngor et al. 2014), with no history of exposure. The liver was the organ used to generate all tissue RNA extracts, except for the Nile tilapia from the health inspection in Florida in which kidney–liver–spleen tissues were pooled by individual.

The known-exposed reference group also incorporated RNA tissue extracts derived from a range of field settings. Thirty-one red tilapia and 14 Nile tilapia (N = 45) samples were collected from various populations in Thailand experiencing TiLV outbreaks at the time of sampling. The sampled tilapia varied in size from fry to broodstock reared in cages within rivers, earthen ponds, or cement ponds reared indoors. More than half (27/45) of these Thai tilapia displayed clinical signs consistent with TiLVD, 16 were subclinical, and the clinical state of 2 fish was not recorded. These 45 samples were confirmed to be TiLV positive by both conventional RT-PCR (Eyngor et al. 2014) and SYBR Green RT-qPCR (Tattiyapong et al. 2018) assays. Samples from additional TiLV field outbreaks, involving Nile tilapia in Peru (N = 1) and Egypt (N = 4) (Nicholson et al. 2017), were included in the known-exposed reference group as they tested positive for TiLV by the same 2 RT-PCR assays. A red tilapia (70 g) from Malaysia (Waiyamitra et al. 2018), a Nile tilapia from Indonesia (800 g) reared in cages within natural waterways, and 2 TiLV isolates recovered from Nile tilapia reared in the USA (Ahasan et al. 2020) were included after they tested positive for TiLV by conventional RT-PCR (Eyngor et al. 2014). A red tilapia cultured in Colombia (12 g), which was sampled during a TiLV outbreak and displayed clinical signs of TiLVD (E. Pulido Bravo & P. Nicholson unpubl. data), was also included as it tested positive by nested RT-PCR (Kembou Tsofack et al. 2017).

2.7. Statistical methods

The difference in mean viral load between tilapia showing clinical signs of TiLVD and those that were subclinical, from the experimental challenge study and the Thailand field outbreaks, were analyzed by comparing the mean Ct values of each group. The Shapiro-Wilk test of normality was used to assess the distribution of Ct values. An independent *t*-test or a Mann-Whitney test was utilized when the data distribution was normal or skewed, respectively. In all analyses, results were considered statistically significant at $p < 0.05$ and confidence intervals for diagnostic sensitivity and specificity were set at 95%. Data were examined by commercial software (IBM SPSS Statistics, version 28).

3. RESULTS

3.1. *In silico* TiLV TaqMan RT-qPCR primer and probe design

The consensus sequence of segment 10, encoding a hypothetical protein, returned the only suitable primers (TiLV-F and TiLV-R) and probe (TiLV-P) combination (Table 1, Fig. 1). An *in silico* analysis of the primers and probe combination for the developed TiLV RT-qPCR assay revealed no mismatches for

Table 1. Primers and probe sequences used in the tilapia lake virus (TiLV) TaqMan RT-qPCR and endpoint PCR assays

Primer/probe name	Sequence (5′–3′)	Melting temp. (°C)	Position in gene (5′–3′)	Amplicon size (nt) including primers
TiLVstdF	TGAGTGTGGCAGATTATTTGTCA	59.2	2–24	303
TiLVstdR	CGGAAAATCGAGATAGGTCACTC	62.8	282–304	
TiLV-F	GGCAAGAAAGCTGCTTCAAAGA	56.3	91–112	64
TiLV-R	CGCTCTCGTCAGCACCATAC	58	135–154	
TiLV-P	CGAAGTTGGAAGAATG	45	115–130	

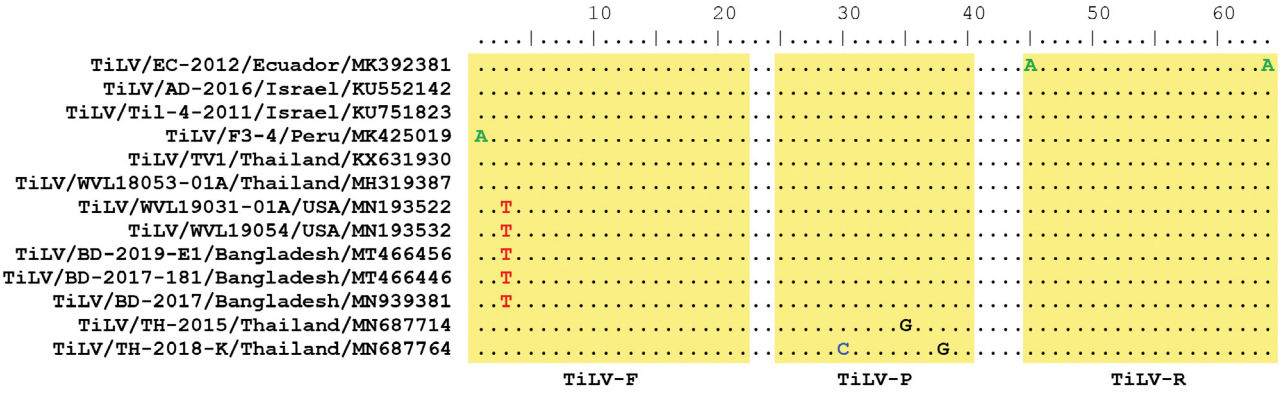

Fig. 1. Alignment of partial (64 bp) segment 10 sequences for 13 tilapia lake virus (TiLV) strains illustrating the *in silico* specificity of the TaqMan RT-qPCR primers (TiLV-F and TiLV-R) and TaqMan probe (TiLV-P). TiLV strains were identified by a unique identifier, the country of isolation, and the associated GenBank accession number

many TiLV isolates (Thailand = 7, Colombia = 1, Israel = 2, Indonesia = 1), 1 mismatch for some TiLV strains (Egypt = 2, Malaysia = 1, Thailand = 1, Peru = 1, USA = 2, Bangladesh = 3), and 2 mismatches for a few TiLV strains (Ecuador = 1, Egypt = 1, Thailand = 1) (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d152p147_supp.pdf).

3.2. Estimation of TiLV TaqMan RT-qPCR assay parameters

The amplification plot revealed that the RT-qPCR assay was linear over 7 orders of magnitude (10^7–10^1 copies) (Fig. 2A). The mean parameters (±SD) for the RT-qPCR assay averaged over the 19 experiments (plates) were as follows: slope = –3.41 ± 0.08, *y*-intercept = 40.93 ± 0.67, R^2 = 0.996 ± 0.002, and efficiency = 96.71 ± 3.20% (Fig. 2B). The LOD of the assay (analytical sensitivity) was determined to be 10 copies of TiLV cRNA (positive in 52/57 reactions, or 91.2% of the reactions). The CV of intra-and inter-assay mean Ct values ranged from 0.18 to 1.41% and from 0.21 to 2.21%, respectively (Table 2). For the analytical specificity, the previously tested positive samples for an orthomyxovirus (infectious salmon anemia virus), a betanodavirus (red-spotted grouper nervous necrosis virus), a picornavirus (clownfish picornavirus), and a rhabdovirus (infectious hematopoietic necrosis virus) were all negative. The IPC was positive for all samples, and the Ct values ranged between 29.41 and 32.17, indicating that PCR inhibitors were not present in the RNA extracts.

3.3. TiLV challenge study

Between 7 and 22 d post virus exposure, tilapia in the low and high dose treatments exhibited clinical signs of TiLVD, including lethargy, gill pallor, cutaneous hemorrhages, ascites, liver pallor, enlarged gall bladder, splenomegaly, and hemorrhages in the brain. Mortality began at 7 d post virus exposure and continued until the trial was terminated on Day 22 with cumulative mortality of 75% (15/20) and 90% (18/20) in the low and high dose treatments, respectively. All 10 negative control fish appeared healthy throughout the experiment. One fish from each of the low and high dose treatments was found dead and determined to be too autolyzed for downstream processing for virus isolation or testing using the TiLV TaqMan RT-qPCR assay. TiLV was isolated from the

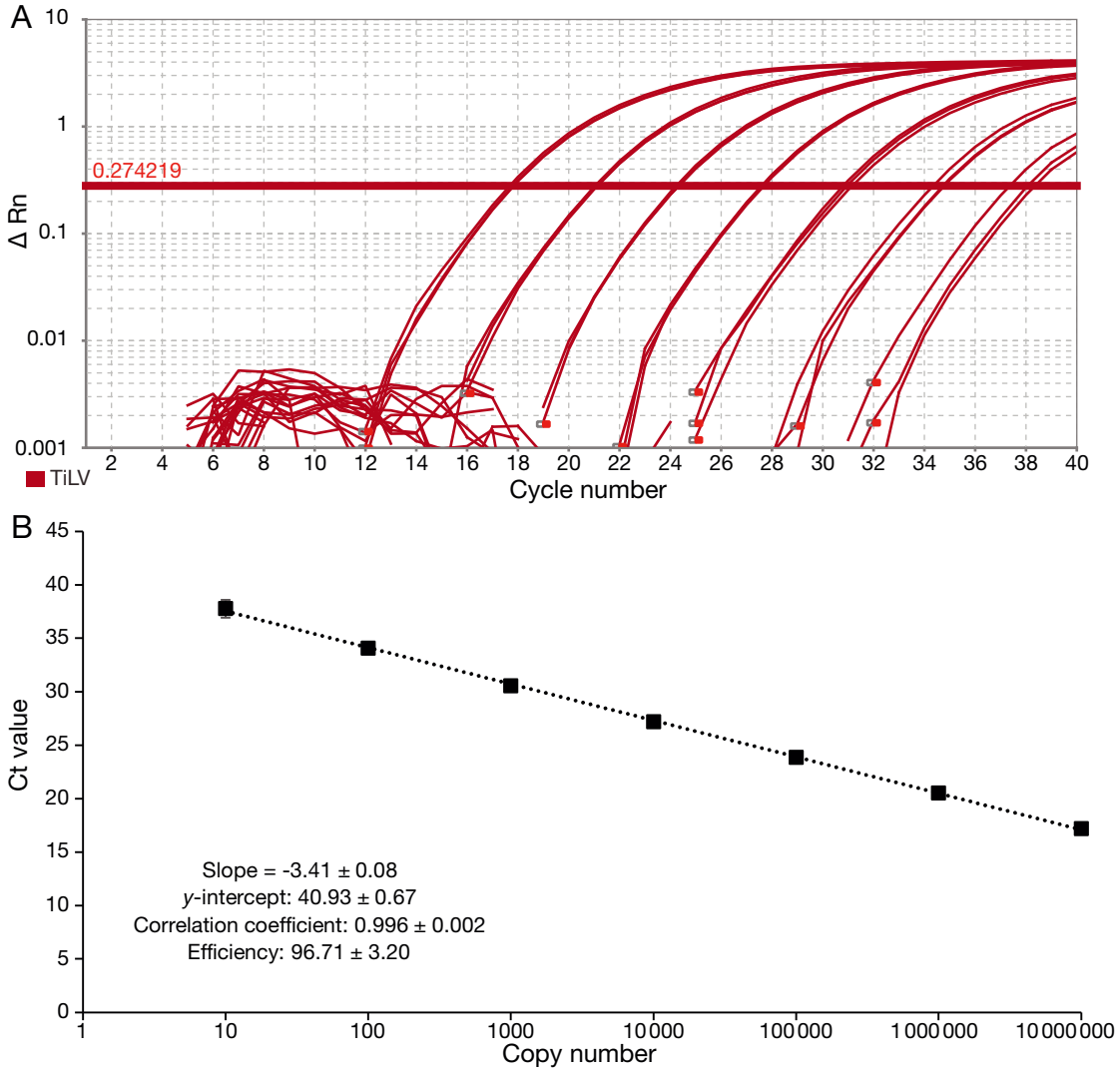

Fig. 2. Tilapia lake virus (TiLV) TaqMan RT-qPCR assay (A) amplification plot and (B) standard curve generated using triplicate 10-fold serial dilutions of the TiLV complementary RNA (cRNA) standard ranging from 10^7 to 10^1 copies. In (A), the red curves indicate amplification of individual TiLV TaqMan RT-qPCR assays. The horizontal red line indicates the automatic threshold assigned by the Applied Biosystems software. The normalized reporter (Rn) is calculated as the ratio of the fluorescence emission intensity of the reporter dye (FAM) divided by the fluorescence emission intensity of the passive reference dye (ROX). The ΔRn is the magnitude of the signal generated during the PCR at each time point as determined by the following equation: ΔRn = (Rn+) – (Rn). Rn+ is the Rn value of a reaction containing all components, including the template, and Rn is the Rn value of an unreacted sample. In (B), the mean RT-qPCR assay parameters (±SE) averaged over the 19 experiments (plates) are provided. Ct: cycle threshold value

liver of 16/19 tilapia in the high dose treatment, 12/19 tilapia in the low dose treatment, and none (0/10) of the negative control tilapia. Of the samples positive for virus isolation, CPE was observed on the initial passage for all samples except 1 sample generated from the low dose treatment that only displayed CPE following the blind passage. Thus, 28/38 tilapia injected with TiLV were positive by virus isolation, resulting in a diagnostic sensitivity of 73.7% (95% confidence limits: 56.6–86.0%). Supernatants from all samples displaying CPE were positive using the TiLV TaqMan RT-qPCR assay. Samples that did not show CPE after blind passage were confirmed to be negative using the RT-qPCR assay. TiLV was detected by RT-qPCR in the liver RNA extracts of 19/19 tilapia in the high dose treatment, 16/19 tilapia in the low dose treatment, and none (0/10) of the negative control tilapia. Among the 10 virus-injected fish that were negative by virus isolation, 5 generated high Ct values (range 33.20–39.24), 2 generated low Ct values (14.18 and 19.04), and 3 samples also tested negative using the TiLV TaqMan RT-

Table 2. Inter-assay (reproducibility) and intra-assay (repeatability) of the tilapia lake virus (TiLV) TaqMan RT-qPCR assay. To determine reproducibility, the reactions for each complementary RNA (cRNA) standard (10^7–10^1 copies) were run in triplicate across 19 experiments (plates). To determine repeatability, data obtained in a single representative TaqMan RT-qPCR plate using a cRNA standard (10^7–10^1 copies) in triplicate are shown. Ct: threshold cycle number; CV: coefficient of variation

Standard dilution	Inter-assay reproducibility				Intra-assay repeatability			
	Ct		CV	No. of wells	Ct		CV	No. of wells
	Mean	SD	(%)	positive (n = 57)	Mean	SD	(%)	positive (n = 3)
10^7	17.21	0.11	0.62	57	17.71	0.08	0.48	3
10^6	20.55	0.04	0.21	57	21.01	0.04	0.18	3
10^5	23.85	0.09	0.36	57	24.23	0.06	0.24	3
10^4	27.18	0.10	0.35	57	27.57	0.05	0.18	3
10^3	30.57	0.15	0.49	57	30.92	0.17	0.55	3
10^2	34.08	0.49	1.44	57	34.55	0.25	0.72	3
10^1	37.77	0.84	2.21	52	37.85	0.53	1.41	3

qPCR assay. The majority of these virus isolation negative samples were derived from the survivors (7/10 samples).

3.4. Estimation of TiLV TaqMan RT-qPCR assay diagnostic sensitivity and specificity

In total, 93 TiLV proven-positive RNA extracts and 185 TiLV proven-negative RNA extracts were used to estimate the diagnostic performance (Table 3). Among 93 TiLV-positive RNA extracts, 90 samples tested positive by the current TaqMan RT-qPCR assay, indicating a diagnostic sensitivity of 96.8% (95% confidence limits: 90.9–99.3%). Diagnostic specificity of 100% (98.1–100%) was generated after 185 TiLV-negative RNA extracts all tested negative.

3.5. Difference in viral load between clinically diseased and subclinically infected tilapia

Using an independent *t*-test, we found that the mean viral loads of fish with clinical signs (mean: 82 048 404 viral genome copies) were significantly higher than surviving fish (mean: 31 viral genome copies) in the experimental challenge study (*t*-test, $t_{33} = -25.736$, p = 0.001). For the samples originating from field outbreaks in Thailand, we calculated the statistical difference between the viral load of the same 2 groups (clinically diseased vs. subclinically infected) using a Mann-Whitney test. Again, tilapia displaying clinical signs of disease had higher viral loads (mean: 24 171 293 viral genome copies) as compared to those with subclinical infections (mean: 8 786 247 viral genome copies) (Mann-Whitney U = 108, p = 0.007).

4. DISCUSSION

The availability of rapid, cost-effective, and validated molecular diagnostic assays capable of detecting TiLV has become increasingly important given the global emergence and impact of TiLV strains (WOAH 2022). In this study, a TiLV TaqMan RT-qPCR targeting a conserved region of segment 10 of the TiLV genome was developed, validated, and shown to successfully detect TiLV in tilapia tissue RNA extracts derived from TiLVD field outbreaks in South America (Colombia, Peru), Africa (Egypt), and Asia (Indonesia, Malaysia, Thailand) (Table 3). In contrast to the TiLV samples tested in this study from Colombia, Egypt, USA, Indonesia, and Malaysia, the tested samples from Peru and Thailand were not specifically tied to the TiLV partial sequences presented in Fig. S1. Compared to previously developed TiLV RT-PCR and RT-qPCR assays, our study included more samples collected from disparate geographic regions to generate validation data for the TiLV TaqMan RT-qPCR (Eyngor et al. 2014, Dong et al. 2017, Kembou Tsofack et al. 2017, Mugimba et al. 2018, Tattiyapong et al. 2018, Waiyamitra et al. 2018). The TaqMan RT-qPCR assay also detected TiLV RNA in infected Nile tilapia liver tissue extracts following an experimental challenge study with a TiLV strain isolated from diseased Nile tilapia in the USA (Ahasan et al. 2020). Finally, the TaqMan RT-qPCR assay successfully detected TiLV RNA in SSN-1 (E-11 clone) cell cultures displaying CPE following their inoculation with TiLV-infected tissue homogenates. Thus, the validated TaqMan RT-qPCR assay should be useful for both research and diagnostic purposes.

An *in silico* analysis of the primers and probe combination for the developed TiLV TaqMan RT-qPCR

Table 3. Description of the tilapia lake virus (TiLV) proven-positive and TiLV proven-negative samples used to estimate the diagnostic performance of the TiLV TaqMan RT-qPCR. SYBR Green RT-qPCR (Tattiyapong et al. 2018); conventional RT-PCR(Eyngor et al. 2014); nested RT-PCR (Kembou Tsofack et al. 2017). IC: intracoelomic

Origin	Sample Type	Sample number	Initial tests	Initial tests positive	RT-qPCR (current study) positive	Reference
Florida, USA	Infection trial	38	TiLV IC injection	38	35	Current study
Florida, USA	Infection trial	10	Sham IC Injection	0	0	Current study
Florida, USA	Health inspection	175	Conventional RT-PCR	0	0	Current study
Thailand	Field outbreak	45	SYBR Green RT-qPCR & conventional RT-PCR	45	45	W. Surachetpong & P. Nicholson (unpubl. data)
Peru	Field outbreak	1	SYBR Green RT-qPCR & conventional RT-PCR	1	1	W. Surachetpong & P. Nicholson (unpubl. data)
Egypt	Field outbreak	4	SYBR Green RT-qPCR & conventional RT-PCR	4	4	Nicholson et al. (2017) (our Fig. S1, GenBank acc. nos. ON099425, ON099426, ON990427)
Colombia	Field outbreak	1	Nested RT-PCR	1	1	E. A. Pulido Bravo & P. Nicholson (unpubl. data) (our Fig. S1, GenBank acc. no. OL539829)
Malaysia	Field outbreak	1	Conventional RT-PCR	1	1	Waiyamitra et al. (2018) (our Fig. S1, GenBank acc. no. OL539827)
Indonesia	Field outbreak	1	Conventional RT-PCR	1	1	W. Surachetpong & P. Nicholson (unpubl. data) (our Fig. S1, GenBank acc. no. OL539828)
USA	Field outbreak	2	Conventional RT-PCR	2	2	Ahasan et al. (2020) (our Fig. S1, GenBank acc. nos. MN193522, MN193532)

assay revealed between 0 and 2 mismatches for 24 TiLV strains from different geographic localities (Thailand, USA, Colombia, Peru, Ecuador, Israel, Egypt, Indonesia, Malaysia, and Bangladesh) (Fig. S1). Primer and probe mismatches can affect assay performance, including assay efficiency (Clark et al. 2018, Stilwell et al. 2018). Mapping of the probe and/or primer sequences to the same 24 TiLV strains revealed a higher number of mismatches for previously developed RT-PCR (Eyngor et al. 2014), nested RT-PCR (Kembou Tsofack et al. 2017), SYBR Green RT-qPCR (Tattiyapong et al. 2018), and TaqMan RT-qPCR (Waiyamitra et al. 2018) assays (Figs. S2–S4). The robustness of the newly developed TaqMan RT-qPCR assay for disease diagnostics was confirmed with the positive results of the isolates from Egypt and Malaysia, even though mismatches were detected. In addition, while another TaqMan RT-qPCR assay (Waiyamitra et al. 2018) could not detect TiLV in Nile tilapia tissues samples from Egypt, our assay successfully confirmed the presence of the virus. Similarly, the Colombian sample was positive by both a nested RT-PCR (Kembou Tsofack et al. 2017) and the TaqMan RT-qPCR assay presented here, while the same sample tested negative by RT-PCR (Eyngor et al. 2014) and by a different TaqMan RT-qPCR assay (Waiyamitra et al. 2018).

Analysis of the analytic performance of the TiLV TaqMan RT-qPCR assay revealed that it was efficient with a high correlation coefficient, and it was also sensitive, specific, repeatable, and reproducible (Table 2, Fig. 2). The TiLV TaqMan RT-qPCR assay detected 10 copies of *in vitro* transcribed TiLV RNA in 91.2% of the reactions and did not amplify the other tested RNA viruses of fish (infectious salmon anemia virus, red-spotted grouper nervous necrosis virus, clownfish picornavirus, and infectious hematopoietic necrosis virus). The TiLV TaqMan RT-qPCR assay possessed a mean efficiency of 96.7% over a linear range from 10^1 to 10^7 copies of TiLV cRNA standards. The TiLV TaqMan RT-qPCR assay developed in this study more accurately reflects the true analytical performance as compared to previously designed RT-qPCR assays that used plasmid DNA standards (Tattiyapong et al. 2017, Waiyamitra et al. 2018) because our *in vitro* transcribed RNA standards better imitate the RNA genome of TiLV.

The diagnostic performance of the TiLV TaqMan RT-qPCR assay was evaluated using tilapia RNA tissue extracts originating from various TiLVD field outbreaks, a TiLV experimental challenge study, and a health inspection of a tilapia producer. The assay diagnostic sensitivity was 96.8% (95% confidence limits: 89.9–99.1%) and the diagnostic specificity was 100% (97.5–100%) (Table 4). The TiLV TaqMan RT-qPCR developed in this study generated Ct values ranging from 39.22 to 11.74, equivalent to 5 to 537 744 640 viral genome copies, in tilapia tissue RNA extracts originating from TiLV field outbreaks and our TiLV experimental challenge study. Moribund tilapia, originating from field outbreaks in Thailand and our experimental challenge study, had higher viral loads as compared to subclinically infected animals. These data underscore the ability of the TiLV TaqMan RT-qPCR assay to detect TiLV RNA in tissue extracts from fish with high viral loads (e.g. lethal systemic infection) as well as those with low to moderate viral loads (e.g. inapparent infections in individuals with low susceptibility, individuals in an early course of TiLVD, or those in a late course of TiLVD [i.e. recovering]). As expected, highly sensitive molecular assays (e.g. semi-nested RT-PCR and RT-qPCR assays) have been shown to be superior to less sensitive testing methods (e.g. RT-PCR and virus isolation) in detecting TiLV in tissues from fish with inapparent infections (Tattiyapong et al. 2017, Liamnimitr et al. 2018, Senapin et al. 2018, Waiyamitra et al. 2018). Additionally, the presented TiLV qPCR assay returns the clinically relevant viral load of a sample which can assist health professionals in determining the role of TiLV during disease investigations (e.g. high TiLV loads are expected in animals displaying symptoms of TiLVD).

Analysis of our experimental challenge study data set confirmed that virus isolation is less sensitive than the TiLV TaqMan RT-qPCR assay. The TiLV TaqMan RT-qPCR detected viral RNA in certain samples in which virus isolation was negative. This discrepancy can be explained by the expected lower sensitivity of virus isolation as compared to the RT-qPCR assay, that the virus was no longer viable, and/or there were neutralizing antibodies in the sample. Thus, using virus isolation as a sole diagnostic test might result in false negative results in the case of subclinically infected fish (i.e. fish with low viral loads). The 2 samples testing negative by virus isolation and positive by RT-qPCR with low Ct values (i.e. high viral loads) were unexpected results and may have resulted from errors that occurred during virus isolation. The 3 fish that tested negative by both assays may never have become infected with TiLV (e.g. error during injection or fish were refractory to infection at the challenge dose), the virus may have been cleared by the immune system, or the infection was below the LOD of both assays. Some tilapia exposed to TiLV mount an immune response resulting in viral clearance, and these survivors develop immunity that protects them from disease if re-exposed to the virus (Pierezan et al. 2020, Tattiyapong et al. 2020). If these 3 fish never became infected and/or cleared the virus, then we underestimated the diagnostic sensitivity for the TiLV virus isolation and RT-qPCR assays.

Based on the analytic and diagnostic performance of the developed TiLV qPCR assay, we recommend its use and continued validation for the diagnosis and surveillance of this globally emerging viral pathogen. To our knowledge, this is the first study to report both the analytic (stage 1) and diagnostic (stage 2) performance for a TiLV RT-qPCR assay as outlined by the OIE for diagnostic assay validation (OIE 2021). An inter-laboratory ring trial involving 6 laboratories is underway as part of evaluating the reproducibility (stage 3) of the TiLV TaqMan RT-qPCR assay (Subramaniam et al. 2022).

Table 4. Estimation of the tilapia lake virus (TiLV) TaqMan RT-qPCR assay diagnostic sensitivity and specificity

Expected TiLV status	TaqMan RT-qPCR Positive	Negative	Total
Positive	90	3	93
Negative	0	185	185
Total	90	188	278

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